DMD Fast Forward. Published on April 21, 2011 as doi:10.1124/dmd.111.038661




# *In vitro* investigation of the glutathione *S*-transferase M1 and T1 null genotypes as risk factors for troglitazone-induced liver injury

Toru Usui, Takanori Hashizume, Takashi Katsumata, Tsuyoshi Yokoi, and Setsuko Komuro

Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan; TU, TH, TK, SK

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan; TU, TY

**Running title**: GSTM1/GSTT1 defects and troglitazone-induced liver injury

Corresponding author: Toru Usui

Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd.

3-1-98, Kasugade-naka, Konohana-ku, Osaka, 554-0022, Japan

Telephone: +81-6-6466-5299, Fax: +81-6-6466-5491

Email: toru-usui@ds-pharma.co.jp

Number of text Pages: 45

Number of Tables: 4

Number of Figures: 6

Number of References: 35

Number of words in Abstract: 249

Number of words in Introduction: 488

Number of words in Discussion: 1437

Nonstandard Abbreviations: GST, glutathione *S*-transferase; CBL, covalent binding level; CYP, cytochrome P450; DILI, drug induced liver injury; BSEP, bile salt export

pump; rhGST, recombinant human GST; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring; $[M-H]^-$, deprotonated molecule; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; HLA, human leukocyte antigen

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



**Abstract**

The double null mutation of glutathione *S*-transferase, GSTM1 and GSTT1 is reported to influence troglitazone-associated abnormal increases of ALT/AST. However, no non-clinical data have hitherto been reported with a bearing on the clinical outcomes and underlying mechanisms. To investigate whether deficiency in GSTM1 and/or GSTT1 is related to troglitazone hepatotoxicity *in vitro*, the covalent binding level (CBL, an index of reactive metabolite formation) and cytotoxicity of troglitazone and rosiglitazone, another thiazolidinedione but with low-hepatotoxicity, were examined using human liver samples phenotyped for cytochrome P450s (CYPs) and genotyped for GSTM1 and GSTT1. Despite addition of GSH, CBLs of troglitazone and rosiglitazone in human liver microsomes were correlated with CYP3A (or CYP2C8) and CYP2C8 activities, respectively. With addition of recombinant GSTM1, the microsomal CBLs of troglitazone and rosiglitazone decreased. However, the CBLs of troglitazone in GSTM1/GSTT1 wild-type hepatocytes were unexpectedly higher than those in null hepatocytes. Although this discrepancy is not fully explained, the GSTM1 and GSTT1 null mutations increased the cytotoxicity of troglitazone, independent of CYP3A or CYP2C8 activities. Furthermore, a GSH adduct of troglitazone, M2 was detected limited to GSTM1 wild-type hepatocytes. Of clear interest, GSTM1 and/or

GSTT1 null mutation-dependent cytotoxicity, and higher exposure to the reactive metabolite trapped as M2 as for troglitazone, were not observed for rosiglitazone. This might at least partly explain the findings related to clinical hepatotoxicity, suggesting that measurement of GSH adducts or cytotoxicity using GSTM1- and GSTT1-genotyped hepatocytes might offer an important *in vitro* system to assist in better prediction of idiosyncratic hepatotoxicity.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



## Introduction

Troglitazone (Rezulin®, Fig. 1) was the first thiazolidinedione, peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist developed for the treatment of type II diabetes (Fujiwara et al., 1995; Sparano and Seaton, 1998). It was also the first in its class approved by the FDA for marketing in 1997 but was subsequently found to rarely induce severe idiosyncratic hepatotoxicity (Gitlin et al., 1998; Shibuya et al., 1998), which led to its withdrawal from the market in 2000. In the meantime, very rare reports of usually milder and reversible hepatotoxicity have been documented with other thiazolidinediones, like rosiglitazone (Avandia®, Fig. 1) and pioglitazone (Actos®) (Isley, 2003). As a particularly interesting example for research on drug-induced liver injury (DILI), numerous researchers have investigated the mechanisms of troglitazone hepatotoxicity.

Although troglitazone hepatotoxicity was not predicted from tests in conventional experimental animals (Watanabe et al., 1999), some cellular events induced by troglitazone may have contributed to the clinically observed hepatotoxicity, for example: 1) reactive metabolite formation by oxidative cytochrome P450 (CYP) 3A mediated metabolism (Kassahun et al., 2001; Tettey et al., 2001; Yamamoto et al.,

2002; He et al., 2004); 2) inhibition of the hepatic drug transporter, bile salt export pump (BSEP) or organic anion-transporting polypeptide (OATP) by troglitazone sulfate (Funk et al., 2001a; Nozawa et al., 2004); 3) mitochondria-mediated toxicity by unchanged-troglitazone (Masubuchi et al., 2006; Lim et al., 2008); 4) troglitazone-mediated apoptosis (Bae and Song, 2003; Shiau et al., 2005); 5) down-regulation of proinflammatory cytokines in Kupffer cells (Sigrist et al., 2000). While these non-clinical data can not explain the idiosyncratic nature of troglitazone hepatotoxicity, high activity of drug metabolizing enzymes (e.g. CYPs) which form reactive metabolites or low activity of detoxification enzymes (e.g. glutathione *S*-transferases, GSTs) which are responsible for scavenging reactive metabolites are likely to be risk factors. In fact, there is a solitary and notable report with aspect to the idiosyncrasy that double mutation of GSTM1 and GSTT1 was associated with abnormally high levels of ALT/AST after administration of troglitazone on retrospective analysis using clinical samples (Watanabe et al., 2003). However, to our knowledge, no non-clinical data to link with the clinical outcomes and provide more detailed clues to mechanisms have hitherto been reported.

The purpose of the present study was to investigate whether GSTM1 and/or GSTT1 defects are involved in troglitazone hepatotoxicity *in vitro*. Clearly, metabolic activation of a drug to reactive metabolites and subsequent covalent binding to target macromolecules might be a necessary first step in the generation of idiosyncratic drug reactions in many cases (Walgren et al., 2005). We have already reported covalent binding is one risk factor for DILI (Usui et al., 2009). Therefore, the covalent binding levels (CBLs, an index of reactive metabolite formation) of troglitazone and rosiglitazone (as a negative control) were investigated using human *in vitro* liver samples with a diversity of CYP phenotypes or GST genotypes. Furthermore, cytotoxicity was also investigated in order to cast light on the idiosyncrasy of troglitazone hepatotoxicity.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



## Materials and Methods

### Materials

[$^{14}$C]Troglitazone and [$^{14}$C]rosiglitazone were synthesized in-house. Unlabeled troglitazone and rosiglitazone were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Pooled human liver microsomes (mixed gender pool of 50 individuals) and 16 individual human liver microsome samples (Reaction Phenotyping Kit, version 7) were obtained from XenoTech, LLC (Lenexa, KS). Cryopreserved human hepatocytes were purchased from In Vitro Technologies, Inc (Baltimore, MD) or XenoTech, LLC. The activities of major drug-metabolizing enzymes in microsomes and hepatocytes had been measured by the suppliers. Use of human samples in this study was approved by ethics committee of Drug Research Division, Dainippon Sumitomo Pharma Co., LTD. Recombinant human GSTA1 and GSTM1 used in Fig. 2 (rhGSTA1 and rhGSTM1, cytosol isolated from *Escherichia coli* expressing human GSTA1 and GSTM1, respectively) and control cytosol (isolated from *Escherichia coli* host strain) were purchased from Cypex LTD (Dundee, UK). rhGSTA1, rhGSTM1 and rhGSTP1 used in Supplemental Figure. 2 were purchased from PanVera Corp. (Madison, WI). To prepare negative control of PanVera rhGST, rhGSTM1 was heat-inactivated at 90°C for 2 minutes. NADPH and reduced GSH were from Oriental

Yeast Co., Ltd. (Tokyo, Japan), and Nacalai Tesque, Inc (Kyoto, Japan), respectively. All other regents and solvents were of the highest grade commercially available.

Incubation using human liver microsomes

In microsomal assays, radio-labeled troglitazone and rosiglitazone (final concentration: 10 μM) were incubated with 1 mg/mL pooled human liver microsomes or 1 mg/mL of the 16 individual human liver microsome samples phenotyped for CYP activities in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 hr in 500 μL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4). rhGSTA1, rhGSTM1, rhGSTP1 or control cytosol (0.4 mg/mL) was added as required.

Genotyping for GSTM1 and GSTT1 using human hepatocytes

GSTM1 and GSTT1 genotyping was carried out by PCR amplification of genomic DNA as described previously (Arand et al., 1996). Genomic DNA was isolated from human hepatocytes using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany).

Incubation using cryopreserved human hepatocytes for measurement of CBL and metabolite analysis

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



Radiolabeled compounds (10 μM) were incubated with cryopreserved human hepatocytes ($1\times10^6$ cell/mL) genotyped for GSTM1 and GSTT1 at 37°C for 8 hrs under an atmosphere of 95%air/5%$CO_2$ in 300 μL suspensions of hepatocytes in incubation medium (XenoTech, LLC).

Measurement of CBL

CBL was measured according to the method we previously reported (Usui et al., 2009). Reactions of microsomes and hepatocytes were stopped by adding ice-cold methanol. For measurement of radioactivity bound to proteins, the reaction mixtures after precipitation were loaded onto glass fiber filters, and washed with 80% (v/v) methanol and acetonitrile to remove unbound radioactivity. The first filtrate was used for radio high performance liquid chromatography (HPLC) analysis or liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of metabolites unbound to proteins as described below. The filter was transferred to a scintillation vial with 10% SDS, and incubated overnight at 55°C to dissolve proteins. The radioactivity and protein concentration were then measured. The CBL was calculated from the following equation:

CBL (pmol equivalent of drug/mg protein) =

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



$$\frac{\text{Radioactivity in the protein solution (dpm/mL)}}{\text{Specific radioactivity of substrate (dpm/pmol)} \times \text{Protein concentration in the protein solution (mg/mL)}}$$

Radio HPLC and LC-MSMS analyses

The first filtrate from the glass fiber filters was collected and evaporated to dryness and the residue was dissolved in the mobile phase and loaded onto a column Inertsil ODS-3 (3 μm, 2.1 ID×150 mm) (GL Science, Inc., Tokyo, Japan) with a column temperature of 40°C. The LC system consisted of an Agilent 1200 (Agilent technologies, Inc., CA) pump set at a flow rate of 0.25 mL/min. The mobile phase to detect GSH adducts of troglitazone consisted of a linear gradient of solvent A (0.1 % formic acid) and solvent B (acetonitrile) according to the following program: B (%): 20 (0 min)-30 (5 min)-35 (26 min)-100 (30 min). The mobile phase to investigate metabolite profiling of troglitazone consisted of a linear gradient of solvent A (0.1 % formic acid) and solvent B (acetonitrile) according to the following program: B (%): 20 (0 min)-30 (5 min)-35 (35 min)-40 (55 min)-100 (65 min). The mobile phase to investigate metabolite profiling of rosiglitazone consisted of a linear gradient of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile) according to the following program B

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



(%): 5 (0 min)-35 (20 min)-60 (35 min)-100 (40 min). Radioactivity was detected with a flow scintillation detector (Radiomatic 610TR; PerkinElmer, Inc, Waltham, MA), using Ultima Flo-M scintillation cocktail (PerkinElmer). Mass analysis was conducted on a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems) equipped with an electrospray ion source for detection of GSH adducts of troglitazone, M1 and M2 (Fig. 1). Ionization parameters included an ion spray voltage of -4100 V and source temperature of 200°C. M1 and M2 were identified by means of multiple reaction monitoring (MRM) in the negative ion mode (MRM transitions; M1, $[M-H]^-$ (deprotonated molecule) = 745→272; M2, $[M-H]^-$ = 779→272). MRM parameters of M1 and M2 included a declustering potential of -60 V and collision energy of -35 V. Molecular mass of other metabolites of troglitazone and rosiglitazone were determined as described in our previous paper (Usui et al., 2009).

Measurement of ATP levels in hepatocytes

Troglitazone and rosiglitazone were incubated with cryopreserved human hepatocytes ($1\times10^5$ cell/mL) genotyped for GSTM1 and GSTT1 at 37°C for 2 hrs under an atmosphere of 95%air/5%$CO_2$ in 100 µL suspensions of hepatocytes in incubation medium (XenoTech, LLC). ATP levels were measured using a CellTiter-Glo

Luminescent cell viability kit from Promega (Madison, WI). This assay generates luminescent signals by luciferase reactions that are proportional to the amount of ATP present.

Statistical analysis

All statistical analyses were performed using SAS Enterprise Guide 4.1 (SAS Institute, Cary, NC). Correlations between CBLs or unchanged-drug amounts and specific CYP activities in human liver microsomes or hepatocytes were analyzed using linear regression analysis. The two-way analyses of variance were used to test the effect of GSTM1 genotype and GSTT1 genotype on CBLs or on remaining unchanged-drug amounts. Student's *t*-test was used to compare the cytotoxicity of troglitazone and rosiglitazone between GSTM1/GSTT1 null and wild-type hepatocytes, and to compare the CBL or M1 amounts with addition between rGST and control cytosol.

## Results

Correlation between CBLs and CYP activities of human liver microsomes from individuals

CBLs of troglitazone and rosiglitazone with 16 individual human liver microsomes phenotyped for enzymatic activities of CYPs were investigated in the presence of 1 mM GSH. The average absolute values were 297 and 435 pmol/mg protein, respectively. Despite addition of GSH as a scavenger, individual microsomes showed large variation in CBLs. Compared to the individual CBLs of troglitazone (152-529 pmol/mg), those of rosiglitazone (141-1013 pmol/mg) varied more widely. The coefficient of variation (CV, %) of CBL was 38% for troglitazone, and 46 % for rosiglitazone. Correlations between CBLs and CYP activities are shown in Table 1. CBLs of troglitazone were significantly and positively correlated with CYP3A activities ($R = 0.89$, $p < 0.001$, testosterone 6β-hydroxylation; $R = 0.83$, $p < 0.001$, midazolam 1'-hydroxylation). Furthermore, CBLs of troglitazone were also positively correlated with CYP2C8 activities ($R = 0.60$, $p = 0.01$). Meanwhile, CBLs of rosiglitazone were significantly and positively correlated with CYP2C8 activities ($R = 0.65$, $p = 0.005$).

Effects of rhGSTA1 or rhGSTM1 on CBLs in human liver microsomes

The scavenging effects of rhGSTA1 or rhGSTM1 (from Cypex LTD) on CBLs of troglitazone and rosiglitazone mediated by pooled human liver microsomes were investigated in the presence of 1 mM GSH (Fig. 2). With addition of rhGSTA1 or rhGSTM1, microsomal CBLs of troglitazone and rosiglitazone were significantly decreased compared with addition of control cytosol. Furthermore, the GSH adduct of a troglitazone reactive metabolite reported as 5-glutathionyl-thiazolidine-2,4-dione (M1, Fig. 1) in a previous publication (He et al., 2004) was increased by addition of rhGSTA1 or rhGSTM1 in radio-HPLC analysis (Representative radio-chromatogram of troglitazone was shown in Supplemental Figure. 1A). rhGSTA1 and rhGSTM1 did not affect the decrease in unchanged-troglitazone in radio-HPLC analysis (data not shown). Meanwhile, no appreciable GSH adducts of rosiglitazone were found in radio-HPLC.

Correlation between CBLs and GSTM1/GSTT1 genotypes in individual human hepatocytes

Fifty-nine individual human hepatocytes were genotyped for GSTM1 and GSTT1 null mutations. Three each were selected for each of the four genotypes of GSTM1/GSTT1

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



(wild/wild, wild/null, null/wild, null/null) (total 12 lots). Using the genotyped 12 individual human hepatocytes, CBLs of troglitazone and rosiglitazone were investigated (Fig. 3). Characterizations of individual hepatocytes about drug-metabolizing enzymes are shown in Table 2. CBLs of troglitazone were higher than rosiglitazone in most of the hepatocytes used (Figs. 3A and 3B). CBLs of troglitazone varied widely and independently of the activities of CYP3A and CYP2C8 (Fig. 3C), which are involved in reactive metabolite formation of troglitazone in microsomes (Table 1). CBLs of rosiglitazone in hepatocytes correlated with CYP2C8 activities ($R = 0.68$, $p = 0.02$) (Fig. 3D). In contrast to the result of microsomal assay using rhGSTM1, CBLs of troglitazone in GSTM1 or GSTT1 null hepatocytes tended to be lower than in GSTM1 or GSTT1 wild-type hepatocytes (Fig. 3A). As the results of two-way analyses of variance to consider the interaction term (Table 3), both GSTM1genotype and GSTT1 genotype significantly affected CBLs of troglitazone ($F = 54.73$, $p < 0.001$ and $F = 6.44$, $p = 0.03$, respectively) without the interaction term. On the other hand, neither GSTM1genotype nor GSTT1 genotype significantly affected CBLs of rosiglitazone ($F = 0.47$, $p = 0.51$ and $F = 0.02$, $p = 0.90$, respectively) without the interaction term.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



Correlation between remaining unchanged-drugs and GSTM1/GSTT1 genotypes in individual human hepatocytes

Remaining unchanged-drug amounts of troglitazone and rosiglitazone were investigated by radio-HPLC analysis from filtrates after 8 hrs incubation with 12 individual human hepatocytes. Representative radio-chromatograms of troglitazone and rosiglitazone are shown in Supplemental Figures.1B and 1C. Remaining unchanged-troglitazone amounts in GSTM1 null hepatocytes tended to be higher than in GSTM1 wild-type hepatocytes (Fig. 4A). As the results of two-way analyses of variance (Table 4), GSTM1 genotype significantly affected remaining unchanged-troglitazone amounts ($F$ = 8.25, $p$ = 0.02) without the interaction term. Remaining unchanged-troglitazone amounts between GSTT1 null and wild-type hepatocytes were not significantly different ($F$ = 2.85, $p$ = 0.13). On the other hand, neither GSTM1genotype nor GSTT1 genotype significantly affected remaining unchanged-rosiglitazone amounts ($F$ = 0.00, $p$ = 0.99 and $F$ = 2.54, $p$ = 0.15, respectively) without the interaction term (Fig. 4B and Table 4). The stability of [$^{14}$C]troglitazone and [$^{14}$C]rosiglitazone under these assay conditions were investigated by radio-HPLC. Troglitazone and rosiglitazone were stable at 10 μM or 50 μM in hepatocyte incubation medium for 8 hrs. Saha et.al. (2010) have been reported that

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



troglitazone sulfate was stable under conditions similar to those in our study, and thus troglitazone sulfate was not considered to be deconjugated to unchanged-troglitazone.

Troglitazone GSH adducts (M1 and M2) in GSTM1- and GSTT1-genotyped hepatocytes and substrate selectivity for the GSH adducts with the GST isoforms

GSH adducts of troglitazone were detected by LC-MS/MS analysis from filtrates after incubation with 12 individual human hepatocytes genotyped for GSTM1 and GSTT1 (Fig. 5). The most notable, M1, was identified with all genotypes. However, M2, which is a GSH conjugate of isocyanate or isothiocyanate, these latter involving a novel oxidative scission of the thiazolidinedione ring system (Fig. 1) (Kassahun et al., 2001), was scarcely obtained with GSTM1 null hepatocytes. No appreciable GSH adducts of rosiglitazone were found with any of the genotypes in this study. The substrate selectivity for troglitazone reactive metabolites trapped as M1 or M2 was verified by incubation of troglitazone with human liver microsome using rhGSTA1, rhGSTM1, and rhGSTP1 from PanVera Corp. in the presence of 1 mM GSH. As the results of LC-MS/MS analysis for detection of GSH adducts, M1 and M2, by means of the MRM method, all used isoforms (rhGSTA1, rhGSTM1, and rhGSTP1) increased the

formation of M1. On the other hand, only rhGSTM1 increased the formation of M2 (Supplemental Figure. 2).

Cytotoxicity assays in individual human hepatocytes

Data for ATP levels in individual cryopreserved human hepatocytes, genotyped for GSTM1 and GSTT1, after treatment with troglitazone and rosiglitazone are summarized in Fig. 6. CYP3A and CYP2C8 activities, which affect reactive metabolite formation of troglitazone and rosiglitazone (Table 1), were comparable among the hepatocytes in this assay (Table 2). Treatment of GSTM1 and GSTT1 null hepatocytes with 50 μM troglitazone markedly reduced ATP levels as compared to no drug control. In contrast, no such reduction was evident in GSTM1 and GSTT1 wild-type hepatocytes exposed to troglitazone. Detected ATP depletion was independent of UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) activities (Table 2). Rosiglitazone did not change the cellular ATP concentration with any of the genotypes.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



## Discussion

"Metabolic idiosyncrasy" and/or "immune idiosyncrasy" are believed to cause DILI (Uetrecht, 2009). Troglitazone-induced liver injury that is not associated with any fever, rash, eosinophilia, or antidrug antibodies (non-allergic hepatotoxicity) has been considered to belong to the category of metabolic idiosyncrasy. Inter-individual differences in drug-metabolizing enzymes derived from polymorphisms that lead to greater exposure to reactive metabolites may be one possible explanation for the idiosyncratic nature of this non-allergic hepatotoxicity (Russmann et al., 2010). Conventional animal experiments would not be expected to be able to reproduce troglitazone hepatotoxicity because of the lack of the genetic variation. In this study, we therefore focused on the metabolic idiosyncrasy of troglitazone hepatotoxicity and tried to investigate the mechanism with human *in vitro* samples, featuring high genetic diversity.

In 16 individual microsomal experiments, CBLs of troglitazone were significantly correlated with CYP3A or CYP2C8 activities despite addition of GSH, which is a scavenger of reactive metabolites (Table 1). Our results are in line with the previous report of formation of troglitazone reactive metabolites through oxidation by CYP3A

(Kassahun et al., 2001; Tettey et al., 2001; Yamamoto et al., 2002; He et al., 2004). However, CBLs of rosiglitazone were also significantly correlated with CYP2C8 activities and both the absolute values and the variability were greater than with troglitazone. Therefore, the risk of reactive metabolite formation alone may not explain the clinical outcomes of troglitazone hepatotoxicity.

It has been reported that GSTM1 and GSTT1 null mutations might cause ALT/AST elevation by troglitazone from an earlier clinical study (Watanabe et al., 2003). Therefore, we expected that low activity of detoxification enzymes, GSTs may be risk factors. rhGSTT1 is not commercially available but addition of rhGSTA1 or rhGSTM1 resulted in decreased microsomal CBLs of troglitazone and rosiglitazone (Fig. 2), suggesting that GSTA1 and GSTM1 potentially has a scavenger effect on reactive metabolites. Regarding the main GST isoforms, GSTA1 and GSTP1, null mutation or polymorphism-related clinical hepatotoxicity is unknown. Thus, although rhGSTA1 also potentially had the scavenger effect, CBLs of troglitazone and rosiglitazone in GSTM1- and GSTT1-genotyped hepatocytes were investigated (Fig. 3). Studied concentrations were determined by reference to estimated unbound maximum concentration in portal vein ($C_{in, max, u}$) of troglitazone, 1.4 μM (calculated in

Supplemental 3). The concentrations of troglitazone and rosiglitazone were determined to be 10 μM to estimate an upper limit of hepatic exposure, and thus the concentration in this assay was considered to be reasonable for evaluation of hepatotoxicity. However, CBLs of troglitazone in GSTM1/GSTT1 null hepatocytes were significantly lower than in GSTM1/GSTT1 wild-type hepatocytes (Fig. 3A and Table 3), independent of CYP3A or CYP2C8 activities (Fig. 3C). GSTs are well known to play crucial roles in detoxification of xenobiotics by preventing the binding of reactive metabolites to cellular proteins and catalyzing the conjugation of electrophilic moieties to GSH (Hayes et al., 2005). Our results were thus contrary to the expectation that GSTs would conjugate and decrease reactive metabolites and subsequent covalent binding, suggesting that microsomal CBL measurements with exogenous rhGSTM1 do not translate to the hepatocyte system with endogenous GSTM1. They are interesting because of the lack of CBL differences between GSTM1/GSTT1 null and wild-type hepatocytes with rosiglitazone (Fig. 3B and Table 3), but unexplained findings, suggesting that not only CBL but also unknown factors are involved in the clinical outcomes of DILI.

In an attempt to clarify the discrepancy, metabolites of individual hepatocytes were analyzed by LC-MS/MS. While M1 was found with all genotypes, M2 was scarcely obtained with GSTM1 null hepatocytes (Fig. 5). As M2 formation is known to be catalyzed by CYP3A (Kassahun et al., 2001) and all used hepatocytes possessed a certain level of basal CYP3A activity (Table 2), it is reasonable to presume that GSTM1 is responsible for scavenging of a reactive metabolite trapped as M2. Verification of substrate selectivity for troglitazone reactive metabolites trapped as M1 or M2 using rhGSTA1, rhGSTM1, and rhGSTP1 (Supplemental Figure. 2) indicated that M1 is formed by any GST isoforms but M2 is formed only by rhGSTM1. It would therefore be possible to suggest that the GSH addition to a reactive metabolite to generate M1 might not be catalyzed by GSTM1, but a separate GSH addition to another metabolite to generate M2 could involve GSTM1 in hepatocytes. GSTM1 null hepatocytes specific exposure to the reactive metabolite trapped as M2 might have potential implications for understanding the relationship between troglitazone hepatotoxicity and GST genotypes in the clinical setting. Remaining unchanged-troglitazone amounts in GSTM1 null hepatocytes were significantly higher than in their GSTM1 wild-type counterparts (Fig. 4A and Table 4). As troglitazone is known to be a time-dependent inhibitor of CYP3A (Kassahun et al., 2001), scavenging

of reactive metabolites that bind to CYP3A protein by GSTM1 and maintaining the metabolic activity of troglitazone elimination could be a possible explanation for the discrepancy in CBL findings between exogenous rhGSTM1 and endogenous human GSTM1.

The cytotoxicity of troglitazone was investigated in the GSTM1- and GSTT1-genotyped hepatocytes. CYP3A and CYP2C8 activities affected reactive metabolite formation of troglitazone (Table 1). Additionally, a combination of high CYP3A and UGT activities demonstrates association with low cytotoxicity of troglitazone while low CYP3A with high SULT activity is linked to higher toxicity in human hepatocytes (Hewitt et al., 2002). However, ATP depletion was considered to be detected after treatment of troglitazone in GSTM1 and GSTT1 null hepatocytes specifically (Fig. 6) and independently of CYP2C8, CYP3A, UGT and SULT activities (Table 2). This finding might be supportive evidence for GSTM1- and GSTT1-dependent hepatotoxicity in a clinical context and this cytotoxicity would be expected to decrease the activities of reactive metabolite-forming enzymes and CBLs, providing another possible explanation for the discrepancy in CBL findings.

Furthermore, it is interesting that GSTM1 and GSTT1 null mutation-dependent cytotoxicity were not observed for rosiglitazone.

Troglitazone sulfate, the main troglitazone metabolite eliminated into bile, shows competitive BSEP inhibition with an apparent $K_i$ value of 0.23 μM (Funk et al., 2001b). Treatment of GSTM1 and GSTT1 wild hepatocytes with 50 μM troglitazone did not reduce ATP levels, despite formation of a certain level of troglitazone sulfate (speculated from Supplemental Figure. 1B). Therefore, transporter inhibition might not be related to the cytotoxicity in the present study. By our system, however, the transporter activities were not investigated and it remains to be determined whether clinical hepatotoxicity is caused by cytotoxicity or by transporter inhibition. Meanwhile, unchanged-troglitazone has been reported to cause mitochondrial dysfunction (Masubuchi et al., 2006; Lim et al., 2008). Thus, our results cannot preclude the higher levels of unchanged-drug in GSTM1/T1 null hepatocytes (Fig. 4) may be relevant to mitochondrial dysfunction. However, expression of CYP3A4 with γ-glutamylcysteine synthetase (GCSh) knockdown was shown to produce troglitazone cytotoxicity in a cell-based assay system (Hosomi et al., 2010), providing supportive

information for our finding that high formation of reactive metabolites and low detoxification might be risk factors for troglitazone cytotoxicity.

Currently, CBL is considered one of the most reliable tools to evaluate DILI (Evans et al., 2004; Nakayama et al., 2009; Usui et al., 2009). On the other hand, there are certainly some agents falsely identified as hepatotoxic by CBL approach (Obach et al., 2008). In the case of troglitazone, CBLs of individual human hepatocytes phenotyped for CYPs and genotyped for GSTs were not appropriate predictors for clinical hepatotoxicity, as the apparent inverse relationship for CBLs and cytotoxicity (and lack of CYP correlation) was observed in hepatocytes. Therefore, a cytotoxicity testing system using GSTM1- and GSTT1-genotyped hepatocytes may be more useful than a CBL measuring system using microsomes or hepatocytes. GSTM1/GSTT1 defects were found to relate to DILI with tacrine and carbamazepine in clinical studies (Simon et al., 2000; Ueda et al., 2007). It will now be necessary to investigate our evaluation system using GST-genotyped hepatocytes with more compounds.

Recently, human leukocyte antigen (HLA) haplotypes were reported to be major determinants of DILI (e.g. flucloxacillin and lumiracoxib) (Daly et al., 2009 and Singer

et al., 2010, respectively), but the association between HLA haplotypes and troglitazone hepatotoxicity in the clinical context has not been reported. As the HLA haplotypes were not investigated in the present *in vitro* study, the immune idiosyncrasy in troglitazone cannot be precluded and further study will be necessary.

In conclusion, our results demonstrate that GSTM1 and/or GSTT1 null mutation may cause higher exposure to the reactive metabolite trapped as M2 or direct cytotoxicity of troglitazone, and appear to provide supportive evidence for metabolic idiosyncrasy of troglitazone hepatotoxicity in the clinical context. They are certainly informative for interpretation of mechanisms of troglitazone hepatotoxicity and indicate that measurement of GSH adducts or cytotoxicity using GSTM1- and GSTT1-genotyped hepatocytes might offer an important *in vitro* system to assist in better prediction of DILI.

**Acknowledgements**

We would like to acknowledge the contributions of Masashi Nakao and Dr. Tomoki Omodani in synthesizing radiolabeled compounds in-house. Appreciation should also be expressed to Takeshi Takagaki for his assistance with the statistical analyses and Shinji Kimoto for his assistance with the assays.

**Authorship Contributions**

*Participated in research design:* Usui, Hashizume, and Yokoi.

*Conducted experiment:* Usui.

*Contributed new reagents or analytic tools:* Usui.

*Performed data analysis:* Usui.

*Wrote or contributed to the writing of the manuscript:* Usui, Hashizume, Katsumata, Yokoi, and Komuro.

## References

Arand M, Muhlbauer R, Hengstler J, Jager E, Fuchs J, Winkler L and Oesch F (1996) A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. *Anal Biochem* **236**:184-186.

Bae MA and Song BJ (2003) Critical role of c-Jun N-terminal protein kinase activation in troglitazone-induced apoptosis of human HepG2 hepatoma cells. *Mol Pharmacol* **63**:401-408.

Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, Daly MJ, Goldstein DB, John S, Nelson MR, Graham J, Park BK, Dillon JF, Bernal W, Cordell HJ, Pirmohamed M, Aithal GP and Day CP (2009) HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet* **41**:816-819.

Evans DC, Watt AP, Nicoll-Griffith DA and Baillie TA (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem Res Toxicol* **17**:3-16.

Fujiwara T, Okuno A, Yoshioka S and Horikoshi H (1995) Suppression of hepatic gluconeogenesis in long-term Troglitazone treated diabetic KK and

C57BL/KsJ-db/db mice. *Metabolism* **44**:486-490.

Funk C, Pantze M, Jehle L, Ponelle C, Scheuermann G, Lazendic M and Gasser R (2001a) Troglitazone-induced intrahepatic cholestasis by an interference with the hepatobiliary export of bile acids in male and female rats. Correlation with the gender difference in troglitazone sulfate formation and the inhibition of the canalicular bile salt export pump (Bsep) by troglitazone and troglitazone sulfate. *Toxicology* **167**:83-98.

Funk C, Ponelle C, Scheuermann G and Pantze M (2001b) Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. *Mol Pharmacol* **59**:627-635.

Gitlin N, Julie NL, Spurr CL, Lim KN and Juarbe HM (1998) Two cases of severe clinical and histologic hepatotoxicity associated with troglitazone. *Ann Intern Med* **129**:36-38.

Hayes JD, Flanagan JU and Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmacol Toxicol* **45**:51-88.

He K, Talaat RE, Pool WF, Reily MD, Reed JE, Bridges AJ and Woolf TF (2004) Metabolic activation of troglitazone: identification of a reactive metabolite and

mechanisms involved. *Drug Metab Dispos* **32**:639-646.

Hewitt NJ, Lloyd S, Hayden M, Butler R, Sakai Y, Springer R, Fackett A and Li AP (2002) Correlation between troglitazone cytotoxicity and drug metabolic enzyme activities in cryopreserved human hepatocytes. *Chem Biol Interact* **142**:73-82.

Hosomi H, Akai S, Minami K, Yoshikawa Y, Fukami T, Nakajima M and Yokoi T (2010) An *in vitro* drug-induced hepatotoxicity screening system using CYP3A4-expressing and γ-glutamylcysteine synthetase knockdown cells. *Toxicol In Vitro* **24**:1032-1038.

Isley WL (2003) Hepatotoxicity of thiazolidinediones. *Expert Opin Drug Saf* **2**:581-586.

Kassahun K, Pearson PG, Tang W, McIntosh I, Leung K, Elmore C, Dean D, Wang R, Doss G and Baillie TA (2001) Studies on the metabolism of troglitazone to reactive intermediates *in vitro* and *in vivo*. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem Res Toxicol* **14**:62-70.

Lim PL, Liu J, Go ML and Boelsterli UA (2008) The mitochondrial superoxide/thioredoxin-2/Ask1 signaling pathway is critically involved in

troglitazone-induced cell injury to human hepatocytes. *Toxicol Sci.* **101**:341-349.

Masubuchi Y, Kano S and Horie T (2006) Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. *Toxicology* **222**:233-239.

Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D and Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos* **37**:1970-1977.

Nozawa T, Sugiura S, Nakajima M, Goto A, Yokoi T, Nezu J, Tsuji A and Tamai I (2004) Involvement of organic anion transporting polypeptides in the transport of troglitazone sulfate: implications for understanding troglitazone hepatotoxicity. *Drug Metab Dispos* **32**:291-294.

Obach RS, Kalgutkar AS, Soglia JR and Zhao SX (2008) Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem Res Toxicol* **21**:1814-1822.

Russmann S, Jetter A and Kullak-Ublick GA (2010) Pharmacogenetics of drug-induced

liver injury. *Hepatology* **52**:748-761.

Saha S, New LS, Ho HK, Chui WK and Chan EC (2010) Direct toxicity effects of sulfo-conjugated troglitazone on human hepatocytes. *Toxicol Lett* **195**:135-141.

Shiau CW, Yang CC, Kulp SK, Chen KF, Chen CS and Huang JW (2005) Thiazolidenediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARγ. *Cancer Res* **65**:1561-1569.

Shibuya A, Watanabe M, Fujita Y, Saigenji K, Kuwao S, Takahashi H and Takeuchi H (1998) An autopsy case of troglitazone-induced fulminant hepatitis. *Diabetes Care* **21**:2140-2143.

Sigrist S, Bedoucha M and Boelsterli UA (2000) Down-regulation by troglitazone of hepatic tumor necrosis factor-α and interleukin-6 mRNA expression in a murine model of non-insulin-dependent diabetes. *Biochem Pharmacol* **60**:67-75.

Simon T, Becquemont L, Mary-Krause M, de Waziers I, Beaune P, Funck-Brentano C and Jaillon P (2000) Combined glutathione-S-transferase M1 and T1 genetic polymorphism and tacrine hepatotoxicity. *Clin Pharmacol Ther* **67**:432-437.

Singer JB, Lewitzky S, Leroy E, Yang F, Zhao X, Klickstein L, Wright TM, Meyer J and Paulding CA (2010) A genome-wide study identifies HLA alleles associated

with lumiracoxib-related liver injury. *Nat Genet* **42**:711-714.

Sparano N and Seaton TL (1998) Troglitazone in type II diabetes mellitus. *Pharmacotherapy* **18**:539-548.

Tettey JN, Maggs JL, Rapeport WG, Pirmohamed M and Park BK (2001) Enzyme-induction dependent bioactivation of troglitazone and troglitazone quinone *in vivo*. *Chem Res Toxicol* **14**:965-974.

Ueda K, Ishitsu T, Seo T, Ueda N, Murata T, Hori M and Nakagawa K (2007) Glutathione S-transferase M1 null genotype as a risk factor for carbamazepine-induced mild hepatotoxicity. *Pharmacogenomics* **8**:435-442.

Uetrecht J (2009) Immune-mediated adverse drug reactions. *Chem Res Toxicol* **22**:24-34.

Usui T, Mise M, Hashizume T, Yabuki M and Komuro S (2009) Evaluation of the potential for drug-induced liver injury based on *in vitro* covalent binding to human liver proteins. *Drug Metab Dispos* **37**:2383-2392.

Walgren JL, Mitchell MD and Thompson DC (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit Rev Toxicol* **35**:325-361.

Watanabe I, Tomita A, Shimizu M, Sugawara M, Yasumo H, Koishi R, Takahashi T, Miyoshi K, Nakamura K, Izumi T, Matsushita Y, Furukawa H, Haruyama H and

Koga T (2003) A study to survey susceptible genetic factors responsible for troglitazone-associated hepatotoxicity in Japanese patients with type 2 diabetes mellitus. *Clin Pharmacol Ther* **73**:435-455.

Watanabe T, Ohashi Y, Yasuda M, Takaoka M, Furukawa T, Yamato T, Sanbuissho A, Manabe S (1999) Was it possible to predict liver dysfunction caused by troglitazone during the nonclinical safety studies? *Iyakuhin Kenkyu* **30**: 537-546.

Yamamoto Y, Yamazaki H, Ikeda T, Watanabe T, Iwabuchi H, Nakajima M and Yokoi T (2002) Formation of a novel quinone epoxide metabolite of troglitazone with cytotoxicity to HepG2 cells. *Drug Metab Dispos* **30**:155-160.

**Footnotes**

Address correspondence to: Toru Usui

Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd.

3-1-98, Kasugade-naka, Konohana-ku, Osaka, 554-0022, Japan

Email: toru-usui@ds-pharma.co.jp

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



**Legends for figures**

**Figure 1 Structures of troglitazone, rosiglitazone, and two postulated troglitazone GSH adducts.**

-SG, glutathione

**Figure 2 Effect of rhGSTA1 or rhGSTM1 on CBLs, and a GSH adduct in human liver microsomes.**

Radiolabeled troglitazone and rosiglitazone (10 μM) were incubated with pooled human liver microsomes and with control cytosol (isolated from *Escherichia coli* host strain, left bars), rhGSTA1 (recombinant human GSTA1 expressed in *Escherichia coli*, central bars), or rhGSTM1 (right bars) in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 hr followed by determination of CBLs. The eluates were analyzed by radio-HPLC, and the amounts of M1 (the main GSH adduct of troglitazone in this assay) are shown (●). No appreciable GSH adducts of rosiglitazone were found in radio-HPLC. The CBL and GSH adduct data are the means ± S.D. from three assays. $^{**}p < 0.01$, and $^{***}p < 0.001$; significantly different from control cytosol.

**Figure 3 Comparison of CBLs in GSTM1/GSTT1 null and wild-type hepatocytes.**

Radio-labeled troglitazone (A, C) and rosiglitazone (B, D) (10 μM) were incubated with 12 cryopreserved human hepatocytes genotyped for GSTM1 and GSTT1 null mutations and phenotyped for CYP activity at 37°C for 8 hrs followed by determination of CBLs. Two-way analysis of variance results for GSTM1 genotype and for GSTT1 genotype affecting CBLs were shown in Table 3.

**Figure 4 Comparison of remaining unchanged-drug amounts in GSTM1/GSTT1 null and wild-type hepatocytes.**

Radio-labeled troglitazone (A) and rosiglitazone (B) (10 μM) were incubated with 12 cryopreserved human hepatocytes genotyped for GSTM1and GSTT1 null mutations and phenotyped for CYP activity at 37°C for 8 hrs followed by radio-HPLC analysis of remaining unchanged-troglitazone and rosiglitazone. Two-way analysis of variance results for GSTM1 genotype and for GSTT1 genotype affecting unchanged-drug amounts were shown in Table 4.

**Figure 5 LC-MS/MS chromatograms of troglitazone GSH adducts, M1 and M2, in GSTM1/GSTT1 null and wild-type hepatocytes.**

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



Filtrates after incubation of troglitazone (10 μM) with human hepatocytes genotyped for the GSTM1/GSTT1 null genotypes (lot. A-C, wild/wild; lot. D-F, wild/null; lot. G-I, null/wild; lot. J-L, null/null) at 37°C for 8 hrs were applied to LC-MS/MS for detection of GSH adducts, M1 and M2, by means of the MRM method in the negative ion mode (MRM transitions; M1, $[M-H]^-$ (deprotonated molecule) = 745→272; M2, $[M-H]^-$ = 779→272).

**Figure 6 Effects of GSTM1 and GSTT1 null mutations on cytotoxicity by troglitazone and rosiglitazone.**

Troglitazone and rosiglitazone were incubated with human cryopreserved hepatocytes genotyped for GSTM1/GSTT1 (□ and ○, wild/wild; ■ and ●, null/null) at 37°C for 2 hrs followed by measurement of ATP levels. Data are mean ± S.D. from three assays expressed as percentages of the no drug control values. $p < 0.01$ was considered statistically significant. $^{**}p < 0.01$, and $^{***}p < 0.001$; significantly different from no drug controls.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



**Table 1 Correlation (*R*) between CBLs and CYP activities of human liver microsomes from 16 individuals.**

| CYP activities | CBLs of troglitazone | | CBLs of rosiglitazone | |
|---|---|---|---|---|
| | *R* value | (*p* value) | *R* value | (*p* value) |
| CYP1A2 (7-Ethoxyresorufin *O*-dealkylation) | 0.08 | (0.76) | 0.09 | (0.73) |
| CYP1A2 (Phenaciten *O*-deethylation) | 0.27 | (0.30) | 0.10 | (0.69) |
| CYP2A6 (Coumarin 7-hydroxylation) | 0.22 | (0.40) | 0.0002 | (0.99) |
| CYP2B6 (*S*-Mephenytoin *N*-demethylation) | 0.51 | (0.05) | 0.18 | (0.50) |
| CYP2B6 (Bupropion hydroxylation) | 0.31 | (0.23) | 0.08 | (0.76) |
| CYP2C8 (Paclitaxel 6α-hydroxylation) | 0.60 * | (0.01) | 0.65 ** | (0.005) |
| CYP2C9 (Diclofenac 4'-hydroxylation) | 0.05 | (0.86) | 0.11 | (0.67) |
| CYP2C19 (*S*-Mephenytoin 4'-hydroxylation) | 0.16 | (0.55) | -0.05 | (0.85) |
| CYP2D6 (Dextromethorphan *O*-demethylation) | 0.37 | (0.14) | 0.06 | (0.82) |
| CYP2E1 (Chlorzoxazone 6-hydroxylation) | -0.02 | (0.94) | 0.04 | (0.87) |
| CYP3A4/5 (Testosterone 6β-hydroxylation) | 0.89 *** | (<0.001) | 0.24 | (0.36) |
| CYP3A4/5 (Midazolam 1'-hydroxylation) | 0.83 *** | (<0.001) | 0.21 | (0.43) |
| CYP4A11 (Lauric acid 12-hydroxylation) | 0.07 | (0.80) | 0.39 | (0.13) |

Radiolabeled troglitazone and rosiglitazone (10 μM) were incubated with 16 individual human liver microsomes in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 hr followed by determination of CBLs. Experiments were conducted in duplicate and correlation (*R*) between the mean of CBLs and CYP activities were analyzed.

Significant correlations; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



**Table 2 Characterization of individual hepatocytes for drug-metabolizing enzymes in the CBL determination assay (Lot No. A to L) and the cytotoxicity assay (Lot No. A, B, J and K).**

| Lot No. | GSTM1/GSTT1 genotypes | Enzymatic activity (pmol/$10^6$ cell/min) | | | |
|---|---|---|---|---|---|
| | | CYP3A | CYP2C8 | SULT | UGT |
| A | Wild/wild | 152 | 24 | 31 | 129 |
| B | Wild/wild | 100 | 33 | 9 | 109 |
| C | Wild/wild | 223 | 20 | 16 | 432 |
| D | Wild/null | 102 | 50 | 9 | 108 |
| E | Wild/null | 94 | 16 | 110 | 415 |
| F | Wild/null | 99 | 8 | 29 | 444 |
| G | Null/wild | 197 | 24 | 33 | 540 |
| H | Null/wild | 259 | 31 | 34 | 508 |
| I | Null/wild | 85 | 46 | 8 | 86 |
| J | Null/null | 174 | 24 | 42 | 366 |
| K | Null/null | 82 | 15 | 14 | 118 |
| L | Null/null | 188 | No data | No data | No data |

Activities of CYP3A (formation rate of 6β-hydroxylated testosterone), CYP2C8 (4'-methylhydroxyl tolbutamide), SULT (7-hydroxycoumarine sulfate), and UGT (7-hydroxycoumarine glucuronide) were measured by the suppliers.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



**Table 3 Two-way analysis of variance results for GSTM1 genotype and for GSTT1 genotype affecting CBLs of troglitazone and rosiglitazone.**

| | Troglitazone | | | Rosiglitazone | | |
|---|---|---|---|---|---|---|
| Source | Mean Square | *F* | *p* value | Mean Square | *F* | *p* value |
| GSTM1 | 25034.5 | 57.43 *** | <0.001 | 80.6 | 0.47 | 0.51 |
| GSTT1 | 2806.0 | 6.44 * | 0.03 | 2.9 | 0.02 | 0.90 |
| Interaction | 7.2 | 0.02 | 0.90 | 139.4 | 0.81 | 0.39 |

Significant at *$p < 0.05$ and ***$p < 0.001$.

DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.



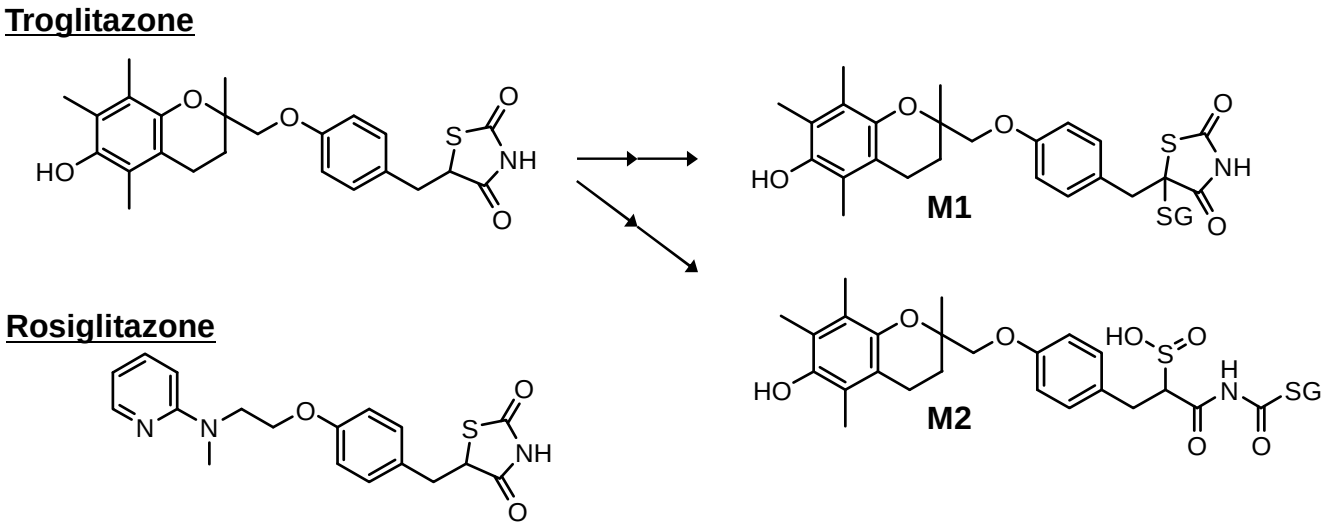
**Table 4 Two-way analysis of variance results for GSTM1 genotype and for GSTT1 genotype affecting remaining unchanged-drug amounts of troglitazone and rosiglitazone.**

| | Troglitazone | | | Rosiglitazone | | |
|---|---|---|---|---|---|---|
| Source | Mean Square | *F* | *p* value | Mean Square | *F* | *p* value |
| GSTM1 | 242.6 | 8.25 * | 0.02 | 0.0 | 0.00 | 0.99 |
| GSTT1 | 83.9 | 2.85 | 0.13 | 47.7 | 2.54 | 0.15 |
| Interaction | 0.1 | 0.00 | 0.96 | 20.9 | 1.11 | 0.32 |

Significant at *$p < 0.05$.

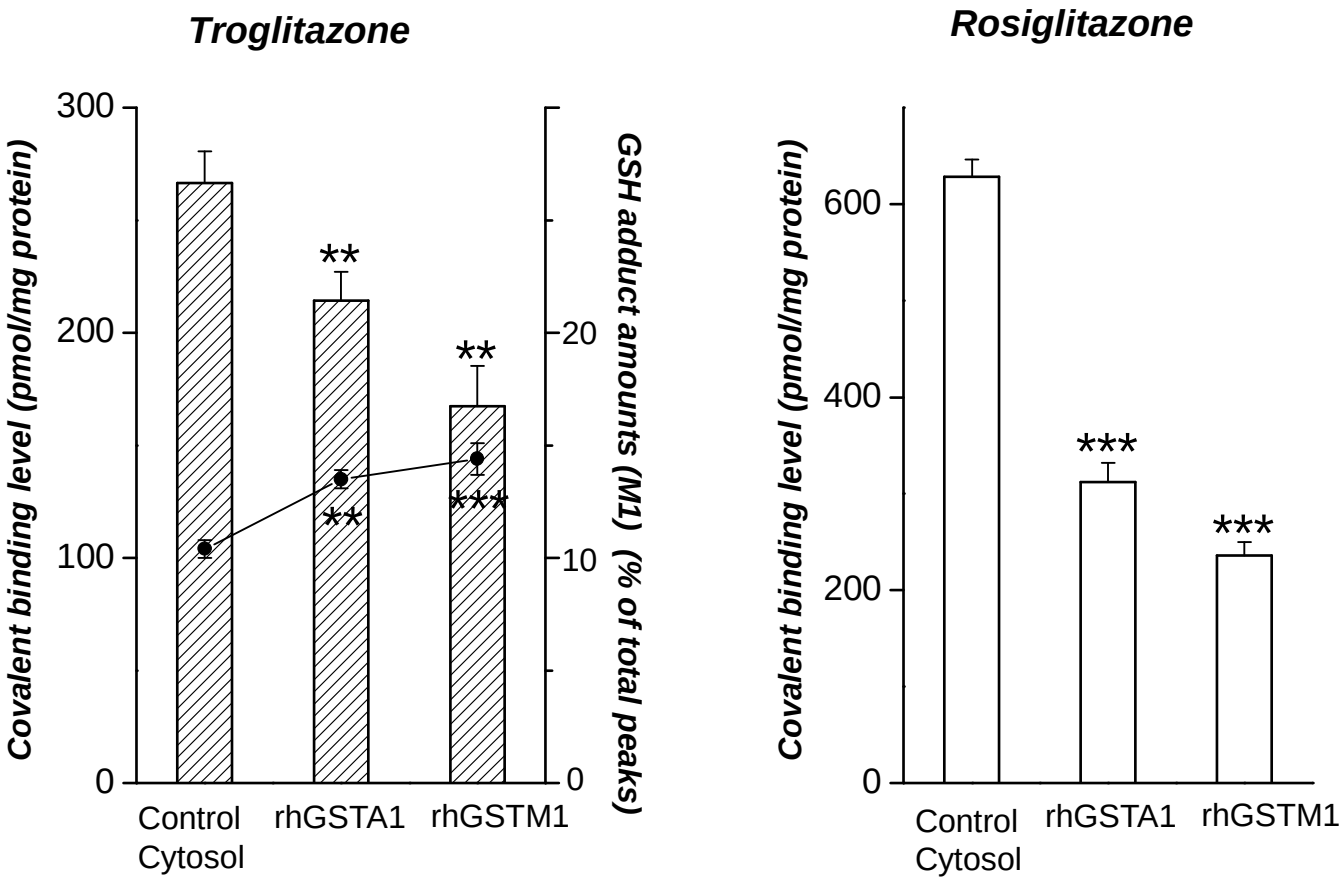
DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

# Figure 1



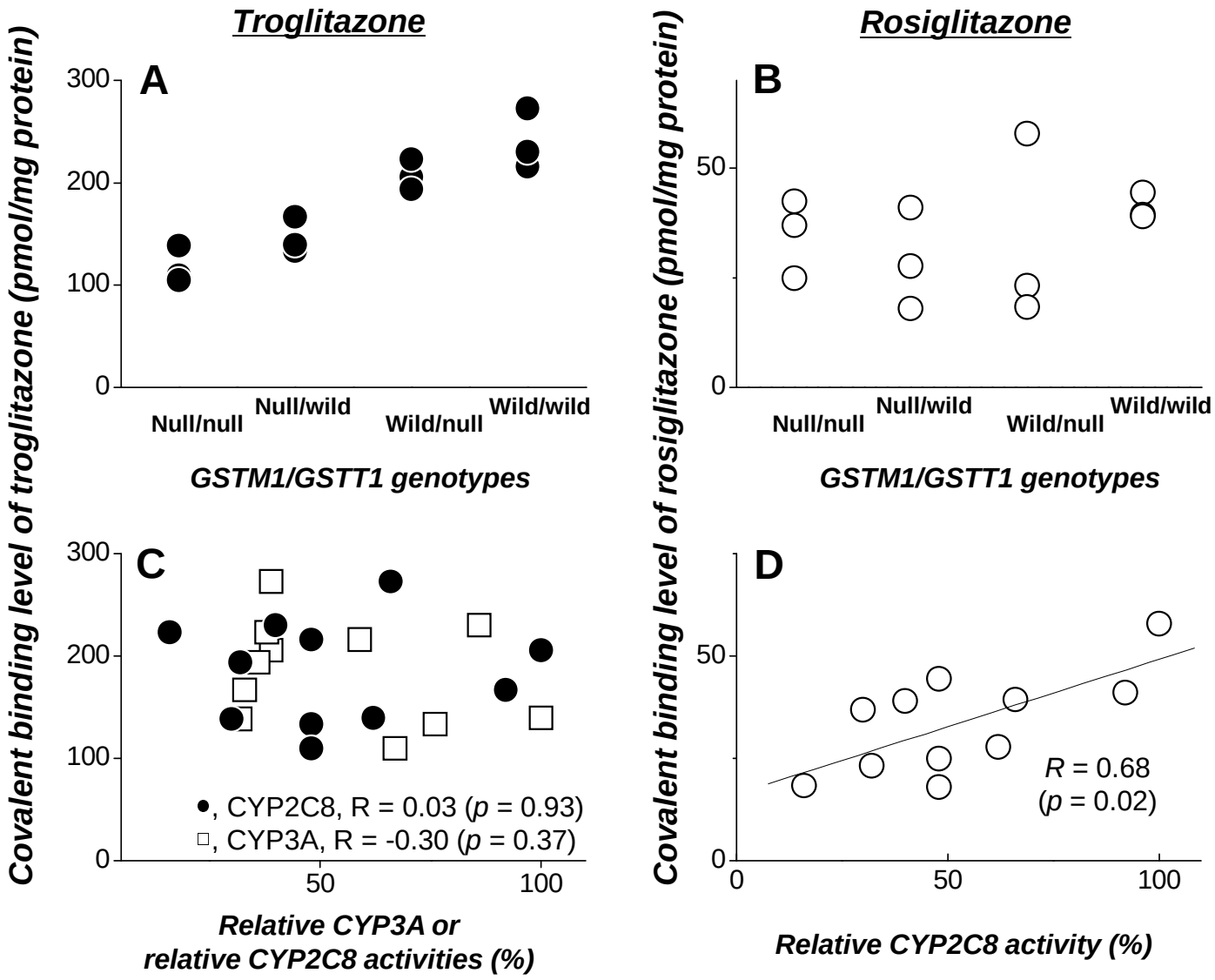
DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 2**



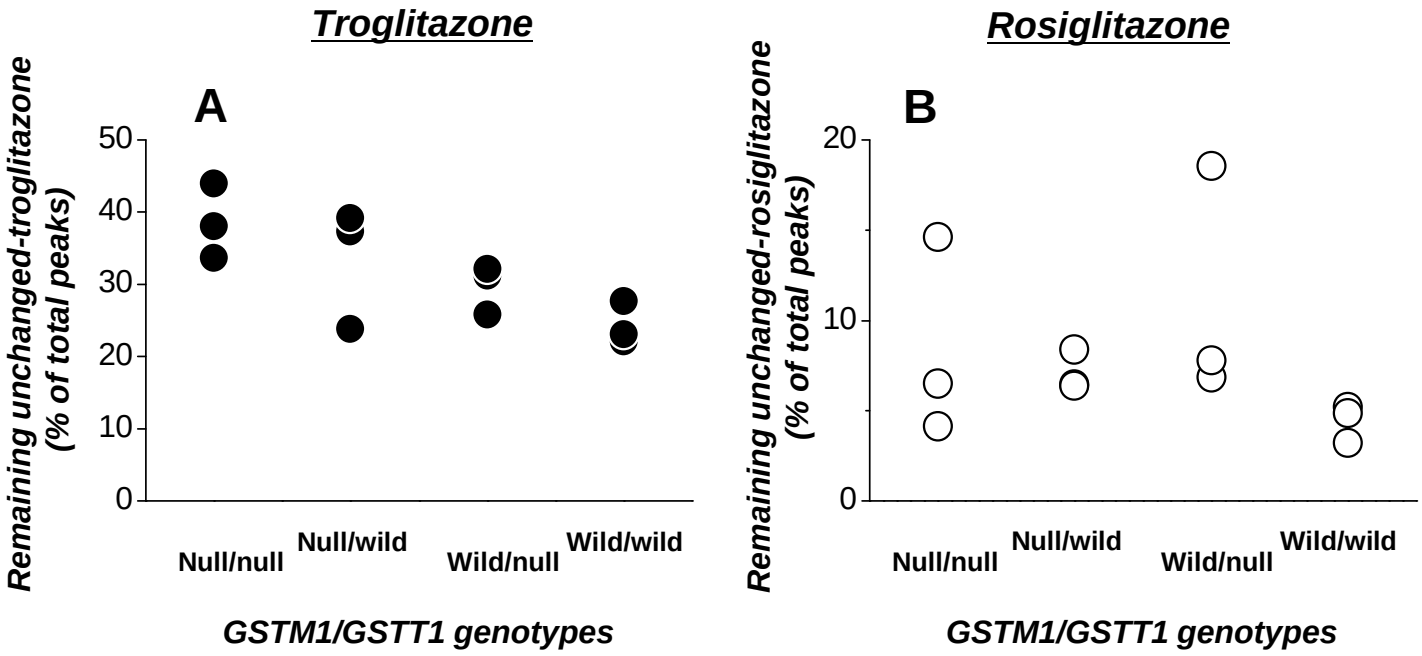
DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 3**



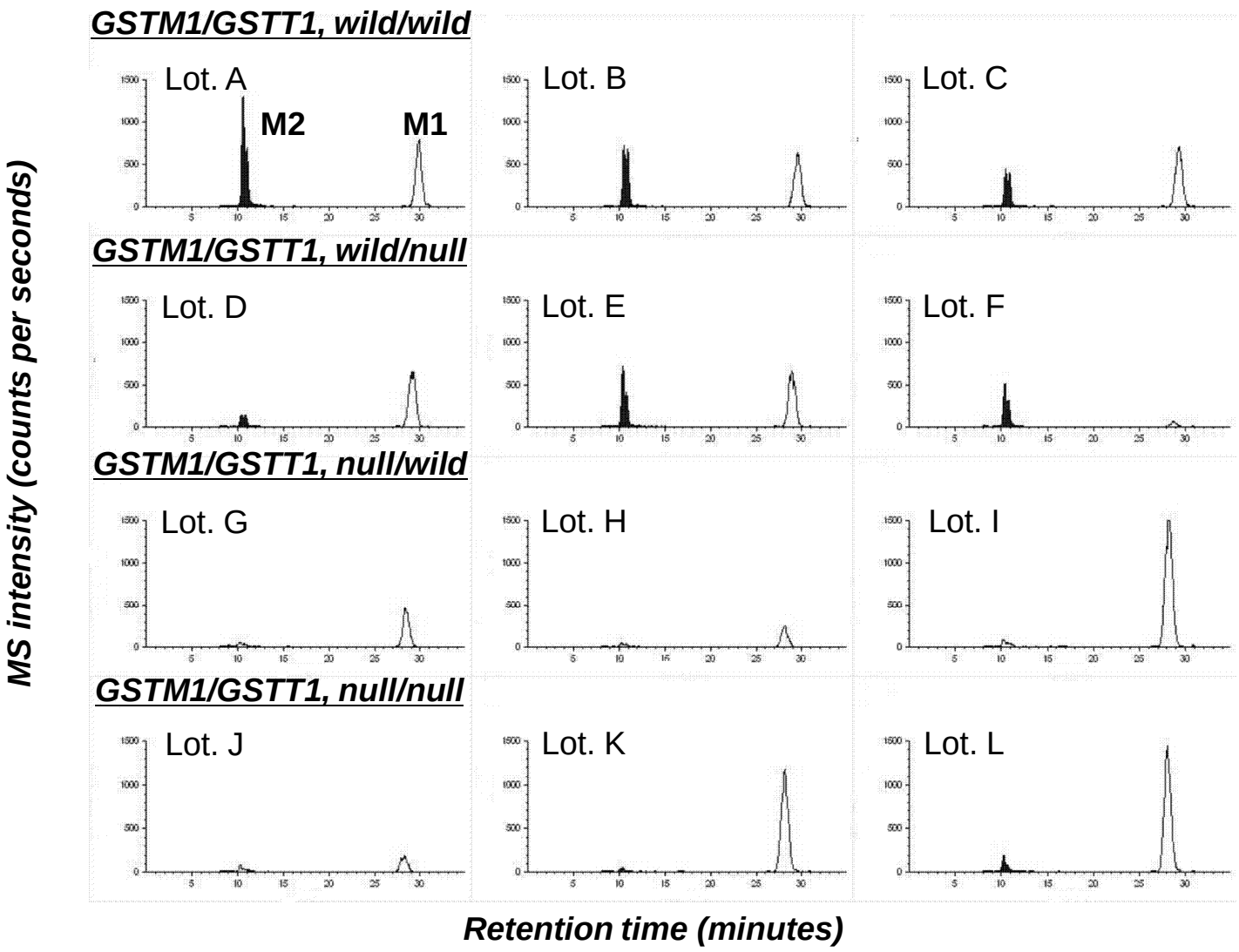
DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 4**



DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 5**



DMD Fast Forward. Published on April 21, 2011 as DOI: 10.1124/dmd.111.038661
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 6**